Polymerase Chain Reaction Products: Analysis Using Capillary Electrophoresis

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Introduction

It is now possible to routinely analyze very small amounts of DNA using a procedure known as the polymerase chain reaction (PCR) [1]. PCR involves repeatedly subjecting a buffered salt solution containing deoxyribonucleotide triphosphates (dNTPs), two strand-specific oligonucleotide primers, a thermalstable DNA polymerase enzyme (Taq), and a small amount of the DNA to be analyzed to a three-step temperature cycle. Using PCR, discrete regions of the source DNA molecules are copied and amplified by the repetitive cycling to readily detectable levels. It is also possible to analyze RNA sequences with PCR after an initial DNA strand (cDNA) and one complementary to it are produced from the original RNA template by the reverse transcription (RT) reaction [2]. The combined process, RT–PCR (also referred to as RNA–PCR), has been effectively used to study small amounts of RNA, such as individual messenger RNAs (mRNAs) or viral RNA, present in tissues and physiological fluids. Both PCR and RT–PCR produce double-stranded DNA (dsDNA) fragments of various sizes which are subsequently isolated and characterized by a number of qualitative and quantitative methodologies.

Discussion

The most common method of analyzing PCR and RT–PCR products involves separating a portion of the reaction mixture by agarose slab gel electrophoresis with ethidium bromide staining to detect the presence of the amplified dsDNA fragments [3]. There are several disadvantages associated with slab-gel techniques, including the following:

1. Gel casting and handling are costly, labor intensive, and not readily automated.
2. A significant portion of the PCR or RT–PCR sample is typically consumed by this mode of analysis.
3. Buffer and reagent consumption, and hazardous waste generated from the use of radioactive probes and ethidium bromide stain, can be considerable.
4. Quantitation requires additional steps and instrumentation for gel imaging and analysis.

During the past decade, capillary electrophoresis (CE)-based techniques have been developed and refined for the analysis of dsDNA products of PCR and RT–PCR [4–7]. CE has several advantages over conventional slab-gel separation techniques, including the following:

1. Capillary electrophoresis instrumentation is fully automated with respect to sample injection, separation, on-capillary detection, and postrun data analysis.
2. Because the separation is conducted in a narrow-bore capillary that facilitates Joule heat dissipation, higher field strengths can be used, resulting in enhanced resolution and shorter run times.
3. Very small amounts (nL) of sample are required for the analysis, thus preserving more of the original sample for subsequent procedures, such as cloning or sequencing.

Considering the expanding base of CE applications, it is becoming clear that the majority, if not all, of conventional slab-gel separation techniques can be readily adapted to capillary format. Analysis of PCR products by CE is becoming more routine and it will likely become one of the primary applications of CE in the area of DNA separations [6]. This entry describes the use of CE-based techniques for the analysis of dsDNA products from PCR and RT–PCR.

Table 1 summarizes selected parameters that are important for establishing a robust and reproducible technique for the separation and quantification of dsDNA products of PCR and RT–PCR using CE. Important advances have been made in a number of areas, including capillary coatings, sieving gel matrices,

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Table 1 Analysis of PCR Products by CE: Selected Technique Parameters

Capillaries
Untreated (bare fused silica): not frequently used
Coated: polyacrylamide, polysiloxane (e.g., DB-1, DB-17), polyvinyl alcohol
Separation Matrix
Buffer: 89–100 m*M* Tris-boric acid, 2 m*M* EDTA, pH 8.2–8.5 (1X TBE)
Sieving gel
Chemical (fixed) gels: cross-linked polyacrylamide, bonded to capillary wall
Replaceable Gels (entangled polymer networks): linear polyacrylamide, methyl cellulose, hydroxypropylmethyl cellulose, hydroxyethylcellulose, polyethylene oxide, polyvinyl alcohol, agarose
Intercalating dyes: 9-aminoacridine (nonfluorescent), ethidium bromide, TOTO, YOYO, YO-PRO-1, TO-PRO-1, TO-PRO-3, SYBR Green I, EnhanCE™
Sample injection
Hydrodynamic: high reproducibility; useful for quantitative analyses; direct injection of untreated samples possible
Electrokinetic: affected by sample salt concentration (i.e., Cl^-); prior dialysis or dilution of the sample required
Detectors
Ultraviolet (UV): 254–260 nm, least sensitive
Laser-induced fluorescence (LIF): up to 1000× more sensitive than ultraviolet
Data analysis
Qualitative: optimization of PCR conditions and product characterization
Quantitative:
Relative: ratio of product (target) to "housekeeping" gene or other heterologous dsDNA internal standard
Competitive: ratio of product (target) to homologous, modified internal standard (competitor); most accurate estimate
Applications
Clinical/diagnostic: screening for genetic abnormalities and diseases
Forensic: human identity testing
Biotechnology: genetic analysis, gene expression, genotyping

and high-sensitivity detection methods. Because of a nearly identical linear negative charge density at neutral pH and above, dsDNA molecules exhibit an electrophoretic mobility in free solution that is independent of molecular size [3]. Therefore, a gel or sieving matrix is required to effect a separation based on molecular size and, for that reason, capillary gel electrophoresis (CGE) has become the specific separation mode most often used for PCR product analysis. Because of the negative charge on DNA molecules, uncoated (bare fused silica) capillaries, which above pH 7 exhibit a strong electro-osmotic flow (EOF) in the direction of the cathode, are rarely if ever used. Instead, capillaries treated with a specific interior surface coating to greatly reduce or completely eliminate EOF are routinely employed in the separation of DNA, a process that is conducted in reversed polarity mode (i.e., cathode at the capillary inlet side). Capillary surface coatings can either be covalently bound to the surface or dynamically adsorbed to the wall. Examples of typical surface coatings include polyacrylamide, polysiloxanes (dimethyl and phenyl–methyl), cellulose derivatives, and polyvinyl alcohol [4–7].

Early CGE separations of dsDNA made use of capillaries in which a polyacrylamide gel was polymerized in and linked to the wall of the capillary, producing what has been referred to as a fixed or chemical gel [4–7]. Although such gels are capable of extremely high resolution due to a well-controlled pore size, they are not commonly used for PCR product analysis because of the problems related to air bubble formation and limited useful lifetime [5]. The development of replaceable sieving gels (also referred to as entangled polymer networks) that can be flushed from the capillary after the separation is complete has been one of the major factors in establishing CGE as a routine method for PCR product analysis. With this system of replaceable gels, a "new"' gel is used for each separation. Some of the most widely used replaceable gel compounds include linear polyacrylamide, alkylcelluloses, polyethylene oxide, agarose, and polyvinyl alcohol [4–7]. These polymer compounds are employed to produce viscous buffered solutions that enable the separation of dsDNA in a capillary based on molecular size [5]. In general, the pore size and, hence, the resolving capacity for dsDNA molecules are controlled by simply manipulating the gel concentration.

One factor that initially hampered the direct analysis of PCR samples by CGE was the presence of high levels of salt, especially chloride ions, in the samples in-

jected into the capillary. Electrokinetic injection, which is the requisite loading method when using fixed gels, is severely affected by the presence of high salt concentration because it impairs the loading of dsDNA into the capillary. Although PCR samples can be injected directly into capillaries using replaceable gels, the presence of salts adversely affects the quality of the results obtained. Also, the presence of other components in the PCR sample (dNTPs, primer oligonucleotides, etc.) not only affect the separation, but they can also obscure peaks when ultraviolet (UV) detection is employed. Fortunately, two relatively simple cleanup methods have been devised to counteract the effects of the PCR sample matrix. The two most common methods are sample microdialysis (float dialysis) and dilution of the sample (20–100-fold) with deionized water prior to CGE [6]. Both methods are effective in reducing the adverse effects of salts, but sample dilution necessitates the use of high-sensitivity detection to compensate for the reduction in the dsDNA concentration.

An important development in CE technology that has helped to promote the analysis of PCR products by CGE is the introduction of laser-induced fluorescence (LIF) detection [4–7]. Because LIF can increase the sensitivity of detection for dsDNA by more than 400-fold over UV detection, it has become the method of choice for the vast majority of dsDNA separations [5]. A practical illustration of the advantage of LIF detection is that typical separations of PCR products by slab-gel electrophoresis with ethidium bromide staining require approximately 5 ng of DNA per band for adequate detection, whereas, with CGE–LIF, subpicogram levels of DNA are readily detected [6].

In order to employ LIF detection, it is necessary to label the dsDNA molecules with a fluorescent compound prior to and/or during their separation by CGE. Two approaches have been employed. The first, and most common, involves the incorporation of an intercalating dye into the separation gel buffer (and, in some cases, into the sample loading buffer), which is highly fluorescent only when bound to dsDNA. Table 1 lists a number of commonly used intercalating dyes. Both monomeric and dimeric dyes have been developed and used to detect PCR products [8]. They offer unique advantages in that they enhance detection sensitivity two to three orders of magnitude over UV detection, and separation resolution and selectivity are often improved with their inclusion [4]. A second approach involves labeling of the primers used in PCR with a fluorophore, such as fluorescein, to produce 5′-end-labeled dsDNA products that can be separated and detected by CGE–LIF. The former approach offers the highest sensitivity with the amount of intercalating dye bound proportional to the size of the dsDNA fragment (i.e., the larger the fragment, the more dye will be bound). Not only do intercalating dyes label the dsDNA for detection, but they also can enhance the selectivity and resolution of dsDNA fragments of similar size [4,6]. Intercalating dyes can also produce anomalous effects on peak shape, depending on such factors as their concentration in the separation or sample buffer and certain sequence-dependent properties of the dsDNA (e.g., %GC composition). These effects result from the binding and retention of differing amounts of dye molecules by the dsDNA fragments during CGE. Therefore, care must be taken to carefully evaluate the use of a specific intercalating dye with a particular PCR product in order to generate reproducible results.

Capillary gel electrophoresis–LIF analysis of PCR and RT–PCR products has been applied to the areas of clinical diagnostics, forensics, and biotechnology. Screening of patients for genetic and infectious diseases, human identity testing using PCR-amplified DNA fragments from specific polymorphic genomic regions (loci) defined by a variable number of tandem repeats (VNTRs) or short tandem repeats (STRs), analysis of mitochondrial DNA, genotyping, and gene expression studies are only a few examples of these applications [4–7]. The types of results that can be gained from CGE analysis of PCR products are twofold. First, CGE is useful in a qualitative evaluation of PCR by separating target DNA from nonspecific products and demonstrating that a single dsDNA fragment resulted from the amplification. CGE is also a rapid method of evaluating various PCR parameters (e.g., cycle number, temperature, $[Mg^{2+}]$, [dNTPs], etc.) for optimizing the efficiency of the reaction. Another use for CGE is to accurately determine the size of the PCR products. This approach has been applied to DNA profiling in human subjects by an assessment of PCR amplified alleles resulting from VNTRs and STRs [6].

The ability to do on-capillary detection and to calculate integrated peak areas from the collected data makes CGE–LIF very useful for the quantification of PCR- and RT–PCR-generated dsDNA products. The quantity of the amplified product can be indicative of the efficiency of PCR and this information can be used to optimize the reaction. Such information is also useful in determining the amount of a specific DNA or RNA present in the analyzed sample. Quantitation can be achieved by relative or absolute estimates. A ratio of target DNA peak area to the peak area of an added dsDNA internal standard gives an estimate of the relative amount of target DNA generated by PCR.

Internal-standard dsDNAs can derive from genes that remain at constant levels in the sample (are unaffected by experimental treatments), such as the so-called "housekeeping" genes or they can be added amounts of a known quantity of a purified and well-characterized dsDNA, such as restriction enzyme digest fragments of genomic DNA. For example, the digestion of ϕX174 bacteriophage DNA with *Hae*III produces 11 distinct dsDNA fragments ranging in size from 72 to 1353 bp [6]. The latter type of standardization also affords the opportunity to accurately determine the size of the PCR product in addition to estimating its quantity when appropriate standards are chosen. Such standards can be obtained from commercial sources.

The most accurate means for quantitation of PCR products, especially those of low copy number, involves a method known as competitive PCR. In quantitative–competitive PCR (QC–PCR), known amounts of an internal standard (competitor) are co-amplified along with an unknown amount of target DNA. The competitor's sequence is chosen to be nearly identical to that of the target except for a small addition or deletion of sequence. The competitor is designed to use the same set of primers as the target so that a competition for them develops. Because the target and competitor are exposed to identical PCR conditions, the ratio of the two products should remain constant even after the reaction has reached its plateau phase. Thus, by plotting the different competitor/target peak area ratios against the amount of added competitor and extrapolating from the point at which the ratio is equal to 1, the amount of target DNA in the original sample can be determined in absolute terms. For RT–PCR, a competitor RNA is used to correct for variable conditions in both the RT and PCR steps [9,10].

Figure 1 depicts a scheme for the estimation of leptin mRNA (encoded by the obese gene, *ob*) contained in total RNA samples isolated from liver and adipose tissues of chickens. Using QC–RT–PCR with CGE–LIF, it was possible to derive absolute estimates (in attomoles) of leptin mRNA. Others have demonstrated a further extension of QC–RT–PCR with CGE–LIF called multiplexing, in which more than one competitor–target pair is subjected to co-amplification and subsequent analysis by CGE–LIF [9,11]. With multitarget QC–RT–PCR, it is possible to monitor PCR-amplified dsDNA corresponding to several genes simultaneously in a single sample assuming PCR conditions have been optimized for each product formed [11].

Future applications of CE to PCR product analysis will arise from improvements in CE instrumentation. Advances in miniaturization of CE devices by producing glass chips with etched channels of <1 cm in length

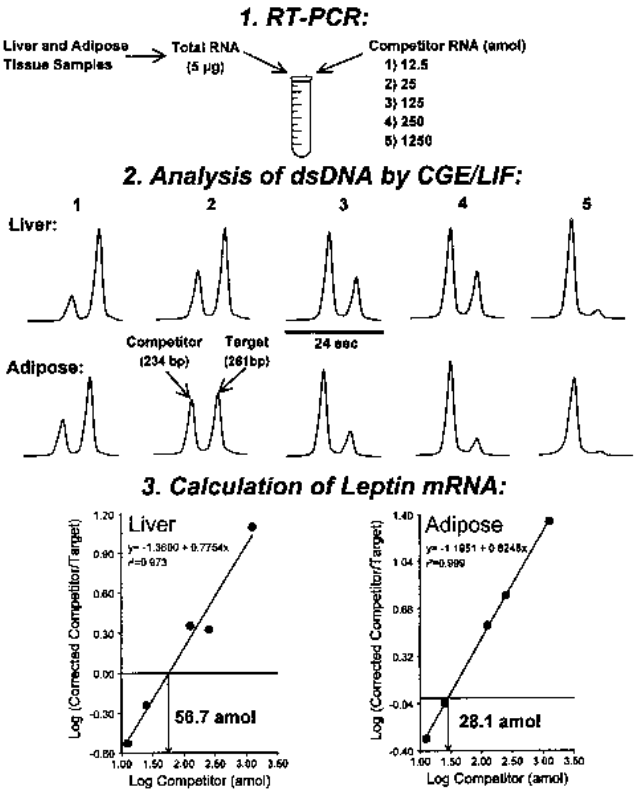

Fig. 1 Analysis of leptin gene expression in chicken liver and adipose tissue by QC–RT–PCR using capillary gel electrophoresis with laser-induced fluorescence detection (CGE–LIF). Target (261 bp) and competitor (234 bp) dsDNA amplicons were separated on a DB-1-coated capillary (27 cm $\times$ 100 μm inner diameter) at a field strength of 300 V/cm in a replaceable sieving matrix consisting of 0.5% HPMC in 1X TBE buffer with 0.5 μg/mL EnhanCE™ intercalating dye. RT–PCR samples (1–2 μL) were diluted 1:100 with deionized water and introduced into the capillary by electrokinetic injection. Separations were completed in under 5 min. A portion (4.4–4.8 min) of each separation shows the changes in the competitor and target peaks. CGE–LIF was more sensitive in detecting both amplicons than agarose slab gel electrophoresis with ethidium bromide staining. The integrated peak area ratio of competitor/target for a series of five individual samples (to which increasing amounts of a synthetic competitor RNA were added prior to RT–PCR) is used to calculate the amount of leptin mRNA (amol) in total RNA isolated from liver and adipose tissue by linear regression analysis.

have already been demonstrated as a feasible method for ultrafast (<45 s) separations of dsDNA [4–7]. The use of multiple capillaries or capillary arrays has proven to be useful in dedicated devices for DNA sequencing [4–7]. Recently, it has been possible to integrate PCR amplification and CE separation in a single

device using an array of eight capillaries for high sample throughput [12]. This technology will undoubtedly produce dedicated CE instruments for PCR product analysis that will feature rapid run time and high throughput. New detection methods such as mass spectrometry offer the promise of increases in selectivity, detection sensitivity, and more accurate quantification of PCR products. It is now clear that, in the future, CE-based analyses of PCR products will continue to get faster and more reliable while achieving wider acceptance by biomedical and biotechnology laboratories.

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